THE ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN PROMOTER REGION -607 C-A OF IL-18 CODING REGION WITH CHRONIC PERIODONTITIS

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Certificate

This is to certify that **Dr. P.SIVSANKAR.**, Post Graduate student (2008-2011) in the Department of Periodontology, Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, has done this dissertation titled **"THE ASSOCIATION OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IN PROMOTER REGION -607 C-A OF IL-18 CODING REGION WITH CHRONIC PERIODONTITIS "** under our direct guidance and supervision in partial fulfillment of the regulations laid down by the **Tamil Nadu Dr.M.G.R. Medical University**, Chennai - 600 032 for **MDS.,** (Branch-II) **Periodontology** (Part II) degree examination.

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ABSTRACT

BACKGROUND: Periodontal disease is a chronic inflammatory disease resulting in destruction of tooth supporting apparatus. Although the disease is microbial in origin destruction of hard and soft tissues of periodontium occurs as an exaggerated host response. 50% variation in inflammatory response/cytokine production is thought to be related to genetic factors such that genetic risk markers are now recognized as important risk determinants of periodontal disease.

IL -18 is a pleiotrophic cytokine with multiple biological activities involved in regulation of both the innate and the aquired immune responses through various biological pathways. The interleukin -18 molecule shares its structural homogeneity with interleukin 1 receptor family, TNF-α. Interleukin -18 plays a role in periodontal disease susceptibility, initiation and progression through the Th1/Th2 cytokine profile.

AIM: The aim of the study is to the distribution of single nucleotide polymorphism, in promoter region -607 C-A of IL-18 coding region, in the study population and to correlate the association of single nucleotide polymorphism (SNP) in promoter region -607 C-A of IL-18 coding region with chronic periodontitis.

MATERIALS AND METHODS: Genomic DNA was obtained from peripheral blood samples in 100 periodontally healthy subjects (control group), 100 subjects with generalized chronic periodontitis (study group) and promoter region -607 C-A of IL-18 coding region was amplified by polymerase chain reaction, and the polymorphism was analyzed by restriction endonuclease cleavage. The alleles were detected by agarose gel electrophoresis and visualised with ethidium bromide.

RESULTS: The percentage distribution of allele frequency for A and C allele among control and study group is statistically insignificant as the p values is greater than 0.05 (p value 0.932). Also the distribution of genotypes for the IL-18 gene polymorphism at position-607 among the control study group is insignificant whose p value is 0.854.

CONCLUSION: Susceptibility for periodontitis is not influenced by the SNP at -607of IL 18 gene that have been investigated in this study and we find no association between them.

CONTENTS

LIST OF ABBREVIATIONS

AAP	-	American academy of periodontology
ANOVA	-	Analysis of Variance
bp	-	base pair
BSA	-	Bovine serum albumin
CAL	-	Clinical attachment level
CD	-	Cluster Differentiation
CP	-	Chronic Periodontitis
COX	-	Cyclo-Oxygenase
DNA	-	Deoxy ribonucleic acid
DZ	-	Dizygous
EBNA	-	Epstein-Barr Virus Nuclear Antigen
ELISA	-	Enzyme linked immunosorbent assay
ETR	-	ets-related protein
GBI	-	Gingival bleeding index
GCF	-	Gingival crevicular fluid
GPI	-	glycosylphosphatidylinositol
GM-CSF	-	Granulocyte-Macrophage Colony-Stimulating Factor
HA	-	Hydroxy Appetite
IKK-1, IKK-2	-	I-κB kinases 1&2
IL-1	-	Interleukin – 1
IL-18RC	-	Interleukin 18 Receptor Complex.
ICE	-	Interleukin 1Beta converting enzyme.
IRAK	-	Il-1 Receptor-Associated Kinase
ISH	-	In situ hybridization
JIA	-	Juvenile Idiopathic Arthritis
MAPK	-	Mitogen Activated Protein Kinase
MEK	-	Mitogen-activated extracellular signal-regulated kinase-activating kinase.
MCP-1	-	Monocytic Chemotatic Protein-1
MHC	-	Major Histocompatiblity Complex

MMP	-	Matrix metalloproteinase
MZ	-	Monozygous
NE Buffer	-	Nuclear extract buffer
NF-IL 6	-	Nuclear factor for IL6
NF-κB	-	Nuclear Factor Kappa B
NIK	-	NF-κB binding kinase
nM	-	Nanomole
PCR	-	Polymerase chain reaction
PGE2	-	Prostoglandin E 2
Pl I	-	Plaque index
pM	-	Picomole
PPD	-	Probing pocket depth
Ras	-	RAt Sarcoma
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
RT-PCR	-	Reverse transcription - polymerase chain reaction
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE buffer	-	Saline EDTA buffer
SNP	-	Single nucleotide polymorphism
TAE	-	Tris base, Acetic acid and EDTA
TBS buffer	-	Tris buffered saline buffer
TE buffer	-	Tris EDTA buffer
TGDW	-	Triple Glass Distilled Water
Th2	-	T helper cell subtype 2
TIMP	-	Tissue inhibitor of metalloproteinase
TRAF6	-	TNF Receptor-Associated Factor 6
RBC lysis buffer	-	Red blood cell lysis buffer
Th1	-	T helper cell subtype 1
Th2	-	T helper cell subtype 2

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INTRODUCTION

Chronic periodontitis is a complex multifactorial disease in which host genetic makeup can modify the course rather than cause it outright[77]. Its aetiology consists of the interplay between anaerobic bacteria found in the biofilm, subgingival dental plaque and exaggerated immune responses in susceptible individuals[95]. Each person has an individual dose–response curve that defines host susceptibility to periodontitis [65,66].

Certain patients are disease-resistant and show no disease progression beyond gingivitis or early periodontitis. This emphasizes the predominant role of host response, rather than bacterial etiology, as the principal determinant of disease expression.

Cytokines play a vital role in the pathogenesis of many of diseases including periodontal disease [71]. Today, vast number of available scientific articles supports the indispensable role of genes in the initiation and progression of periodontal disease by altering the immune responses at various levels. Specifically, different allelic variants can result in variations in tissue structure (innate immunity), antibody responses (adaptive immunity), and inflammatory mediators (non-specific inflammation)[42,95] which could act as protective or risk factors for periodontitis[61], For this reason, periodontitis is considered as a complex genetic disease whose phenotype is determined by both the genetic makeup and the environmental influences on the affected individual.

The twin study design, the most popular and a reliable tool in genetics supports the involvement of genetic components of a disease. This also substantiates the contribution that genes make vs. the environment in a phenotypic expression. Based on twin study design Adult periodontitis was estimated to have 50% heritability, which was unaltered following adjustments for behavioural variables including smoking. It was concluded that there is indeed a substantial genetic basis for the risk of chronic periodontitis[55].

Currently, the presence of interleukin genetic variations appears to identify individuals who are at increased risk for more severe chronic periodontitis and for a less predictable response to therapy.

In 1997, **Kornman et al**[45] described a composite genotype formed by the two polymorphic loci – interleukin-1A 889) and interleukin-1B (+3953) – single nucleotide polymorphisms that carry a C–T transition. This was later known as periodontitis-associated genotype.

IL-18 is a proinflammatory and tumor-suppressive cytokine, which due to its structure, receptor family and signal transduction pathways belongs to the IL-1 cytokine family [9,58]. It is unique, with the capacity to induce either Th1 or Th2 differentiation depending on the immunological context[59]. In the presence of IL-12, IL-18 induces Th1 responses while in the absence of IL-12, it promotes Th2 responses. It is clear, therefore, that IL-18 has the ability to induce either Th1 or Th2 differentiation. It is logical to hypothesize, SNPs in IL-18 coding region may modulate the diseases process.

Recently six different polymorphisms within the IL-18 gene have been identified. Three Single Nucleotide Polymorphisms (SNPs) were found in the promoter region (-656 G/T; -607 C/A, -137 G/C) and two of the polymorphisms were observed in the 50-untranslated region (1113 T/G, 1127 C/T)[28]. Moreover, one polymorphism was found in the coding region of the IL-18 gene at position 105[32]. Although the functional significance of the aforementioned polymorphisms is yet not fully established it was suggested that particularly the polymorphisms at position -607 and -137 are associated with considerable changes in IL-18 expression[28,85].

Functional effective polymorphisms in genes coding for cytokines that are involved in the regulation of the inflammatory process are attractive candidates to delineate the genetic background of periodontitis. Based on this rationale, the present study aimed to assess whether polymorphisms of the IL-18 gene contribute to the pre-disposition to periodontitis and the distribution of genotypes among patients with periodontitis and healthy controls.

AIM AND OBJECTIVE

- To study the distribution of single nucleotide polymorphism, in promoter region -607 C-A of IL-18 coding region, in the study population.
- To correlate the association of single nucleotide polymorphism (SNP) in promoter region -607 C-A of IL-18 coding region with generalized chronic periodontitis.

REVIEW OF LITERATURE

Cytokines an Overview

Cytokines are central to the pathogenesis of an ever-increasing number of diseases, including periodontal disease. They are inter-cellular messengers and, as such, represent a key mechanism by which cells involved in immune responses communicate. Numerous cytokines are produced in response to microbes and other antigens and stimulate diverse responses. Cytokines interact in a network by inducing each other, transmodulating cell-surface receptors, and by synergistic, additive, or antagonistic interactions on cell function[7].

Cells that produce cytokines include the macrophages/monocytes, dendritic cells, lymphocytes, neutrophils, endothelial cells, and fibroblasts[1].

In immune regulation of periodontal disease cytokines are usually produced transiently, often in picomolar concentrations. Indeed, the majorities of immune responses occur locally, and often between two cells conjugated together. In other situations, however, large amounts of cytokine are produced, which allows cells to communicate at a distance. Such cytokines include IL-1 and IL-6, which are produced by a large number of cells and in relatively large quantities[79].

Gemmell and Seymour 2004[25] Postulated that "appropriate" cytokine production results in protective immunity, while "inappropriate" cytokine production leads to tissue destruction and disease progression.

Cytokines and T Cell Subtypes

Several cytokines are involved in Th1 immune responses. These include: IL-12, IL-18, IFN- γ and tumor necrosis factor (TNF) α .In contrast, IL-4, IL-5, and IL-13 are involved in Th2 immune responses and promote humoral immunity by promoting B-cell growth and differentiation[8,57].

O'Garra, 1998[67] established the induction role of cytokines in the development of heterogenous T cell subsets and tremendous built-in redundancy of cytokines, such that many cytokines have overlapping functions. He also postulated that equally, many cytokines are antagonistic, and, again, the overall biological effect is the result of the balance between all cytokines, rather than their individual levels. The balance between cellular and humoral responses, for example, is strongly regulated by the balance between Th1 and Th2 subsets .Th1 cells are characterized by their production of interferon-γ (IFN-γ) and are responsible for directing cell-mediated immune responses leading to the eradication of intracellular pathogens, but may also cause immunopathology and organ-specific autoimmune disease if deregulated.

Interleukin 18-An Overview

IL-18 was first discovered in 1995 and was originally identified as interferon-γ (IFN-γ)-inducing factor[68].

Nakanishi *et al.*, 2001a[59] reported that IL- 18 is unique, with the capacity to induce either Th1 or Th2 differentiation, depending on the immunological context

IL-18 is a proinflammatory and tumor-suppressive cytokine, which, due to its structure, receptor family, and signal transduction pathways, belongs to the IL-1 cytokine family[9,58].

Locus of IL- 18 Gene and Structure

The human IL-18 gene is located on chromosome 11q22; the synthesized protein contains 193 amino acids and shares 64% identity with its mouse homologue structure of IL-18[63].

Biet *et al.*, 2002[9] reported that IL -18 display structural similarities although shares less than 30% primary amino acid sequence homology with the IL-1 family members—(IL-1α, IL-1βand IL- 1 receptor antagonist (IL-1Ra)

Li *et al.*, 2003[49] stated that while IL-1 and IL-18 and their receptors are members of the IL-1 family, there are several biological and functional differences among them. One of these is that IL-18 induces IFN-γ from T-cells and NK cells, but does not cause fever, whereas induction of fever is a well-known characteristic of IL-1.

Kato *et al.* 2003[40] using MNR spectroscopy, have described the IL-18 structure. Its overall structure is well defined, except the segment between residues 34 and 43. IL-18 consists of 12 strands that form 3 twisted four-stranded β sheets, with one short α helix and one 310-helix. The three β sheets are packed against each other, to adopt a β trefoil fold, which is similar to those of IL-1β, but the surface residues are totally dissimilar. The loop, residues 32-42, spreads out from the body of the protein

and seems to be flexible. He further described 3 sites important for receptor activation: two sites that serve as binding sites for IL-18R (α) and the third for binding to IL-18R (β).

IL -18 Synthesis:

Similar to IL-1β, IL-18 is intracellularly synthesized as a 24-kDa biologically inactive precursor (pro-IL-18)[9,17,18,19,27,31,69] and is secreted as an 18-kDa inactive form requiring caspase-1 (ICE) (which is the same enzyme that cleaves pro- IL-1β IL-1β-converting enzyme), to cleave it into the active IL-18 molecule[88].

Nakanishi *et al.*, 2001b[60] suggested that this process could occur after Toll-like receptor-4 (TLR-4) is activated by lipopolysaccharide (LPS), or, alternatively, upon stimulation with FasL, a caspase other than ICE, termed 'casp1-like', which might be activated to cleave the pro-IL-18 into active IL-18.

IL-18 Receptor: Characteristics, Expression and Activation

Parnet *et al.*, 1996[74] reported IL-18R to be selectively expressed on Th1, but not Th2, cells, and was proposed to be considered as a cell-surface marker to distinguish Th1 from Th2 cells

Born *et al.*, 1998[12] IL-18 shares structural organization with the IL-1R/Toll-like receptor (TLR) super family. The IL-18 receptor (IL-18RC) complex consists of 2 receptor chains: the ligand -binding IL-18Rα chain, originally identified as IL-1 receptor-related protein (IL-1Rrp), and the core-receptor IL-18Rβchain, formerly designated as accessory-protein-like (IL-18AcPL). Both chains are required for

signaling. The activity of IL-18 begins with its binding to the IL-18RC. IL-18Rα binds IL-18 with low affinity, whereas IL-18Rβ does not bind IL-18, but increases the affinity of the receptor and participates in signal transduction

Sigal, 2005[80]. IL-18 receptor (IL-18R) is found on T-cells, NK cells, B cells, and dendritic cells.

The mechanism by which the formation of the IL-18RC leads to intracellular signal transduction is as follows the recognition of glycosylphosphatidylinositol (GPI) anchor glycans by IL-18 and IL-18Rα induces formation of the IL-18/IL-18Rα CD48/GPI anchor glycan complex, and this complex and the specific peptide sequences trigger binding to IL-18Rβ and induce intracellular signal transduction[23].

Intra Cellular Signaling by IL-18

Following activation of pro-IL-18, active IL-18 mediates IL-18R aggregation, after which the heterodimeric complex recruits the adaptator molecule MyD88, as well as IL-1 receptor-associated kinase (IRAK). After phosphorylation, IRAK dissociates from the receptor complex and then associates with TNF receptor-associated factor 6 (TRAF6), which leads to the sequential activation of NF-κB binding kinase (NIK), I-κB kinases (IKK-1, IKK-2). This leads to the release of the NF-κB subunits p65/p50 from I-κB, and their translocation to the nuclear κB binding sites. Alternatively, the binding of IL-18 triggers the activation of Ras, resulting in an activation cascade of Raf, mitogen-activated extracellular signal-regulated kinase-activating kinase (MEK), and mitogen activated protein kinase (MAPK). This induces phosphorylation of MAPK. After phosphorylation, MAPK is translocated into the

nucleus and phosphorylates NF-IL6, which allows NF-IL6 to associate to the C/ERP binding site[9,17,39,60,76].

In epithelial cells, IL-18 transduction is primarily *via* the MAPK p38 pathway, rather than NF-κB, which may explain the absence of cyclo-oxygenase (COX)-2 and the inability of IL-18 to cause fever[47].

Biological Activities of IL- 18:

Although Th2 cells expressing IL-18R have been reported[86], they cannot produce IFN-γ in response to anti-CD-3 and IL-18, whereas functional IL-18R is selectively expressed on Th1 and shows a strong capacity to produce IFN-γ in response to anti-CD-3 plus IL-18 or IL-1β.

Reported that IL-18 synergizes with IL-12 to induce IFN-γ production from natural killer (NK) and T-cells and, therefore, Th1 cell development, but does not depend on IL-12 for its activity[9,52,60,68].

Chang *et al.*, 2000[15] reported that IL-18, in the absence of IL-12, induces the production of Th2 cytokines from T-cells, NK cells, and also basophils/mast cells[60]. However, large amounts of IFN-γ, as well as IL-12Rα expression on T-cells are induced in response to IL-12 and IL-18 .Therefore, IL-18 stimulates either Th1 or Th2 responses, depending on its local cytokine milieu.

Comparison of the biological activities of IL-18 and IL-1β is shown in the data[9].

Properties	*IL-18*	*IL-1β*
Activation of Th1 responses	+ (in the presence of IL-12)	±
Activation of Th2 responses	+ (in the absence of IL-12)	-
Synergy with IL-12 for IFN-γ production	+	+
Induction of IFN-γ	+	±
Protection against infection	+	+
Precursor requires ICE cleavage	+	+
Induction of IL-8/IL-1β/TNF-α	+	+
B-cell activation	+	+
Increased expression of adhesion molecules	+	+
Binding to IL-1R	-	+
Binding to IL-18R	+	-
IRAK activation	+	+
NF-κB translocation	+	+

It is evident that IL-18 has the capability to stimulate innate immunity as well as both Th1 and Th2 immune responses[41,52].

In the absence of IL-12, IL-18 has been shown to induce the production of IL-4, IL-5, IL-10, and IL-13 by T- and NK cells, and also prostaglandin E2 (PGE2) production by activated macrophages .IFN-γ is essential for activating macrophage microbicidal activity, as well as promoting subsequent Th1-like immunity[39].

IL-18 therefore enhances IL-12 and IL-2 activities and also those of IL 10, and markedly enhances IFN-γ, IL-1, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF production, making it, like IL-12, a remarkable pro-inflammatory cytokine[52].

While IL-12 and IL-18 play a major role in IFN-γ induction, it has also been shown that PGE2 has the capability to antagonize the potent inductive signal provided by the combination of IL-12 and IL-18. Physiological concentrations of PGE2 significantly suppressed NK cell IFN-γ synthesis, regardless of cytokine stimulation. PGE2 may thus play an important role in limiting the innate and adaptive immune processes through direct suppression of NK cell IFN-γ synthesis[89].

In addition to the above, IL-18 also plays an important role in the activation of non-T-cell populations, such as macrophages, neutrophils, endothelial cells, synovial fibroblasts, chondrocytes, osteoclasts, and keratinocytes[35,48,50,60].

Chemotaxis of human NK cells is enhanced by IL-18, which also induces the production of activated MMP-2 and pro-MMP-2, as well as MT1-MMP, from NK cells[36].

With regard to the B-cells, it has been found that human B cell lines ubiquitously express mRNA for IL-18, IL-18Rα, and IL-18Rβ, but do not secrete IL-18 protein; hence, IL-18 possibly affects B-cells through paracrine actions, and it may well be that B-cells may be passive bystanders with an innate ability to use IL-18[51].

IL-18 promotes Fas-dependent apoptosis of endothelial cells, hepatocytes, and basophils[14,20,78].

Keratinocytes responded to IL-18 with an up-regulation of the major histocompatibility complex (MHC) class I and II expression, and production of the chemokine CXCL10, suggesting that IL-18 may also contribute to the chronicity of inflammatory skin diseases[90].

IL-18 In Infections and Systemic Diseases:

Bohn *et al.*, 1998[11] demonstrated the inhibition of IL-18 production with the use of anti-IL-18 antibodies which lead to uncontrolled bacterial infections. The central role of IL-18 in the development of protective immunity against bacterial infections is well-established.

Fujioka *et al.*, 1999[22] have shown in invitro experiments that IL-18 also has protective properties against several viruses.

There have been several reports relating the up-regulation of IL-18 to human inflammatory and autoimmune diseases, including rheumatoid arthritis[93], psoriasis[44], type I diabetes[62], atherosclerosis[53], and chronic heart failure/coronary heart disease[10,95].

Periodontitis and rheumatoid arthritis are chronic inflammatory disorders that share similar biological mechanisms of tissue destruction. Persons with rheumatoid arthritis display a poorer periodontal status[54].

An incipient attachment loss has been reported[56] more frequently in those with juvenile idiopathic arthritis (JIA) than in control individuals, despite having similar amounts of plaque and marginal bleeding .This study examined neutrophils activity and proinflammatory cytokines in the two groups, and found significantly elevated serum levels of IL-1β and IL-18 in the JIA group. When the JIA group was subdivided according to the presence or absence of attachment loss, IL-18 was significantly increased in the subgroup with attachment loss.

IL 18 and AIDS

Pages *et al.*, 2005[73] has demonstrated that the Epstein-Barr virus nuclear antigen (EBNA2) can induce the expression of the IL-18 receptor (IL-18R), both in Burkitt lymphoma and in non transformed B-cell lines, allowing the infected cells to respond to IL-18. EBNA2 expression has also been associated with IL-18R expression *in vivo* in EBV-positive B lymphomas from persons with AIDS

IL-18 And Periodontal Diseases

In addition to its capacity to act as a potent co-stimulus for Th1 induction, and its ability to induce TNF-α and IL-1β in mononuclear cells, IL-18 is able to initiate a cytokine cascade with a concomitant increased expression of pro-inflammatory markers, such as chemokines, nitric oxide, adhesion molecules, and MMP-9[43,64].

Xu *et al.*, 1998[91] reported IL-18 together with IL-12, in much lower concentrations, had an increased effect on reducing bone-resorbing activity, whereas IL-12 alone had no significant effect. This synergistic effect could be due to the fact that IL-12 increases the responsiveness to IL-18 *via* an increase in IL-18 receptor expression.

Yamada *et al.* 2002[92] investigated the *in vitro* effect of IL-18 on osteoclastic bone resorbing activity, and found that when different concentrations of IL-18 were applied to osteoclast-enriched cell cultures, bone resorbing activity decreased.

Also, the combination of the two cytokines ,IL-18&IL-12 synergistically increased the concentrations of IFN-γ, suggesting the importance of IFN- γ in the inhibition of osteoclastic bone resorbing activity.

Grandjean-Laquerriere *et al.* 2004[29] investigated the ability of HA particles to induce production of active IL-18 by human monocytes. They used 10 HA-based powders that differed in size range, shape, and surface area, and demonstrated that HA particles can trigger IL-18 production in human monocytes. The parameter that demonstrated the greatest influence on the cells proved to be shape. The most significant production of IL- 18 was observed when cells were exposed to needle-shaped particles

Johnson and Serio 2005[38] have reported on the concentrations of IL-2, IL-4, IL-6, IL-10, IL-12, IL-18, and IFN-γ in the gingival tissues of Dominican Republic Hispanic individuals with healthy and diseased periodontium. Diseased periodontal tissues displaying bleeding on probing were subdivided into 3, 4 to 6, and > 6 mm probing depths. Concentrations of all the cytokines adjacent to 4-6-mm diseased sites were greater than in healthy sites, where IL-12 concentrations were higher.

Johnson and Serio, 2005[38] have reported IL-6 and IL-18 concentrations were greater adjacent to > 6 mm sites compared with healthy sites. IL-6 and IL-18 positively correlated with deep probing depths, while IFN-γ and IL-12 demonstrated negative correlations. It was suggested that IL-18 and IL-6 accumulate within the gingiva, possibly contributing to a non-resolving hyper-inflammation mediated by a shift toward a Th2 phenotype.

Orozco *et al.*, 2006[70] suggested that the local production of IL-1β and IL-18 in the gingival crevicular fluid increased with increasing inflammation, and IL-18 was the predominant cytokine at both gingivitis and periodontitis sites. Very little IL-12

was detected, with levels decreasing with increasing inflammation. Therefore, it was suggested that there is an association between the severity of periodontal disease and IL-1, IL-12, and IL-18 levels.

Recently **Pradeep A.R *et al* 2009**[75] has researched wherein sixty subjects (30 males and 30 females; age range: 26 to 49 years) participated in the study. The subjects were initially divided into three groups, consisting of 20 subjects in each group, based on the gingival index, probing depth (PD), clinical attachment loss (AL), and radiologic parameters (bone loss): healthy (group 1), gingivitis (group 2), and periodontitis (group 3), patients in group 3 after treatment constituted group 4. GCF samples were collected from all of the groups to estimate the levels of IL-18 and MCP-1 using enzyme-linked immunosorbent assay. In the result the mean IL-18 and MCP-1 concentrations in GCF were highest in group 3 (330.61 pg/ml and 73.3 pg/ml, respectively). The results suggest that IL-18 and MCP-1 levels increased in GCF from periodontal health to disease and decreased after periodontal therapy. Levels of IL-18 and MCP-1 positively correlated with PD and clinical AL in group 3. In addition, IL-18 and MCP-1 levels significantly correlated with each other in groups 2, 3, and 4. The results were GCF IL-18 and MCP-1 concentrations increased in periodontal disease compared to health and correlated positively with the severity of disease. Further, based on the positive correlation of IL-18 and MCP-1 in this study, they reported that IL -18 may promote an inflammatory response by the induction of MCP-1 production and the subsequent recruitment and activation of circulating leukocytes at the inflammatory site.

Genetic Polymorphisms

Genetic polymorphisms have historically been used as genetic markers to locate disease-causing genes through linkage studies. By definition, a genetic polymorphism is a nucleotide sequence at a particular position in DNA molecules exhibiting at least two structural variants (alleles) that occur in the population at a frequency of greater than 1%. There are a number of different types of nucleotide structure in the human genome that fit this definition.

- Variable Number of Tandem Repeats (VNTR)
- Microsatellite Polymorphism
- Single Nucleotide Polymorphism (SNP)

A single nucleotide polymorphism (SNP)[5] is a variation in the identity of a single nucleotide at a particular site in the genome. The SNP defines two alleles for which there could be three genotypes amongst individuals in a given population. SNPs include, but are not limited to, restriction fragment length polymorphisms (RFLPs), i.e. nucleotide changes that create or destroy sites for restriction endonucleases in the DNA molecule.

Michalowicz et al 1991[55] reported the periodontal condition of 110 pairs of twins from 16 to 70 years old. The mean probing depth and attachment level scores were found to vary less in MZ twins reared together than in DZ twins reared together. In a subsequent related study of 64 MZ and 53 DZ adult twin pairs, Michalowicz and co-workers estimated the genetic and environmental variances and heritability in CP according to path models with maximum likelihood estimation techniques. MZ twins were found to be more similar than DZ twins for all clinical measures. Statistically significant genetic variance was found for both the severity and extent of disease.

Adult periodontitis was estimated to have approximately 50% heritability, which was unaltered following adjustments for behavioural variables including smoking.

Kornman et al 1997[45] reported the correlation between genetic polymorphisms and Chronic Periodontitis undertaken by the IL-1 gene. The combined presence of the R allele in the IL-1α gene at nucleotide position-889 and the R-allele of IL-1α gene at nucleotide position +3953 were associated with severity of periodontitis in non-smoking Caucasian patients.

Giedraitis et al. 2001[28] identified five different polymorphisms within the IL-18 gene (promoter region and untranslated region). Three single nucleotide polymorphisms (SNPs) were found in the promoter region (-656 G/T; -607 C/A, -137 G/C) and two of the polymorphisms were observed in the 50-untranslated region (1113 T/G, 1127 C/T) Moreover, one polymorphism was found in the coding region of the IL-18 gene at position 105[32].

The human IL-18 promoter gene cloned and screened for possible polymorphisms. Three single nucleotide polymorphisms were detected in the promoter and two polymorphisms in the 59-nontranslated region of the gene. Three combinations of these polymorphisms were observed in the Swedish population. All IL-18 promoter alleles were found to have clear promoter activity when inserted into a luciferase reporter vector. There were no significant differences in promoter activity between alleles without stimulation, but after stimulation with PMA/ionomicin one of the alleles had clearly lower activity than the other (P 0.01). Measurement of IL-18 and IFN-γ production in 48 multiple sclerosis patients by RT-PCR showed slightly higher expression of IL-18 in individuals homozygous for the most frequent haplotype. Two IL-18 promoter polymorphisms were analyzed by sequence specific

PCR in 208 multiple sclerosis patients and 139 healthy controls, however, no significant differences were found. The data indicates that common IL-18 promoter polymorphisms influence the expression of IL-18 and potentially also of IFN-γ suggested in their mutation analysis experiments, Although the functional significance of the aforementioned polymorphisms is yet not fully established, the polymorphisms at position -607 and -137 are associated with considerable changes in IL-18 expression[28,86].

Folwaczny M et al 2005[21] reported a total of 123 patients with periodontitis and 121 healthy controls were genotyped for six IL-18 polymorphisms at position -656, -607, -137, 1113, 1127 and codon 35/3. In their study genotyping was performed by PCR and restriction fragment length polymorphism analysis. The frequencies of alleles and genotypes as well as haplotypes within both study groups were compared using the Pearson Chi-square test at a level of significance of 5% (p-0.05). Coseggregation was found to be 100% for the two polymorphisms at position -656 and -607 as well as for the polymorphisms at position -137, 1113, and 1127. The distribution of genotypes for the IL-18 gene polymorphism at position -656/-607 (p50.854), at position -137/1113/1127 (p50.320), and at codon 35/3 (p50.481) was not significantly different among periodontitis patients if compared with healthy control subjects. The distribution of haplotype combinations for the -607 and -137 polymorphism also showed not significant difference between the both study groups (p50.545).They concluded that those six different IL-18 gene polymorphisms were not associated with destructive periodontal disease.

MATERIALS AND METHOD

STUDY DESIGN AND SUBJECT SELECTION:

After passing ethical clearance to conduct the research, around 200 patients in the age group of 30- 70 years who attended the Outpatient wing of Department of Periodontology, Tamilnadu Government Dental College, Chennai during March to December of 2010 participated in the study . The patients were divided into 2 groups, 100 subjects with generalized chronic periodontitis and 100 periodontally healthy subjects as study and control group respectively. All were Tamil speaking Dravidians.

A written informed consent was obtained from all patients. A complete medical, dental history and the following periodontal parameters , plaque index, gingival bleeding index, probing depth, clinical attachment loss were recorded and their ethnic origin was confirmed from their oral history.

About 5 ml of venous blood samples were collected from the patients with or without periodontitis for analyzing their genotypic status and to find the distribution of C-A single nucleotide polymorphism at -607 position of IL-18 promoter region.

Inclusion Criteria:

1. Subjects above or equal to 35 years of age were included for study group and above or equal to 30 years were included for control group.
2. Includes both the gender.
3. Selected samples were the ethnic origin of Tamil speaking Dravidians.

Exclusion criteria:

1. Diabetes or Immunodeficiency syndromes
2. Any disease known to compromise immune function
3. Periodontal treatment within past 6 months
4. Pregnancy or Lactation
5. Premedication for dental treatment
6. Non-steroidal anti-inflammatory drugs in the past three years
7. Immunosuppressive chemotherapy
8. Presence of any acute infection

The subjects selected for the study were divided into two groups, control group and study group of one hundred each based on the above said criteria.

CONTROL GROUP: (Healthy Periodontium):

Control group included 100 subjects with clinically healthy periodontium. Subjects found to exhibit no signs of periodontal disease as determined by the absence of clinical attachment loss and no sites with probing pocket depth >3 mm[4].

STUDY GROUP: (Generalised Chronic Periodontitis):

Study group included 100 subjects (smokers included) with clinical attachment loss ≥1mm[4] involving > 30% of total numbers of sites examined[13].

STUDY PROTOCOL

1. Obtaining approval from Institutional ethical committee.
2. Sample selection by applying inclusion and exclusion criteria
3. Obtaining informed consent
4. Documenting the clinical parameters namely gingival bleeding index, plaque index, probing pocket depth and clinical attachment level.
5. Collection of blood sample
6. DNA isolation and purification
7. Quality check of DNA
8. Polymerase chain reaction
9. Restriction fragment length polymorphism to detect the genotype
10. Statistical analysis
11. Data interpretation.

Following selection of subjects, informed consent was obtained from all the subjects selected for the study after explaining the study procedure verbally. Examination was preceded by a thorough medical and dental history of the subjects.

Intra-oral examination was done using mouth mirror and Williams periodontal probe. Periodontal evaluation was done by measuring the gingival bleeding index, plaque index, probing pocket depth (PPD) and clinical attachment level (CAL).

Clinical Parameters:

Gingival Bleeding index[3] (GBI)

Teeth examined - All teeth

Surfaces examined - 4 sites for each tooth (Mesial, buccal, distal, palatal)

The presence or absence of bleeding is determined by gentle probing of the gingival crevice with a periodontal probe

Criteria for Scoring:

Positive score (+) - Presence of bleeding on probing within 20 seconds

Negative score (-) - Absence of bleeding on probing within 20 seconds

$$\% \text{ of bleeding sites} = \frac{\text{Total number of positive score}}{\text{Total number of surfaces of all teeth}} \times 100$$

PLAQUE INDEX[81] (PlI)

Surfaces Examined

Distofacial, facial, mesiofacial, lingual

Criteria for scoring

Score 0 : No plaque

Score 1 : Film of plaque at marginal area recognized by running a probe

Score 2 : Moderate accumulations as can be seen by naked eye

Score 3 : Abundance of soft matter

Calculation

$$\text{Plaque Index (PI) per tooth} = \frac{\text{Total of score}}{4}$$

$$\text{PI per individual} = \frac{\text{Total of PI per tooth}}{\text{Total no. of teeth examined}}$$

Interpretation

0	-	Excellent
0.1 – 0.9	-	Good
1.0 – 1.9	-	Fair
2.0 – 3.0	-	Poor

Probing Pocket Depth (PPD)[30]

Probing pocket depth is measured from the gingival margin to the base of the pocket using William periodontal probe. The probe is passed under the gingiva along the circumference of the tooth. Three measurements are made on the buccal aspect and three on the lingual aspect of each tooth-total of six sites per tooth (Mesiobuccal, Midbuccal, Distobuccal, Mesiolingual, Midlingual and Distolingual).

Clinical Attachment Level (CAL)[13]

Clinical attachment level is measured from the cementoenamel junction to the base of the pocket using Williams periodontal probe. When the gingival margin is located on the anatomic crown, the level of the attachment is determined by subtracting from the probing pocket depth, the distance from the gingival margin to the cementoenamel junction. If both are the same, the loss of attachment is zero.

When the gingival margin coincides with the cementoenamel junction, the loss of attachment equals the probing pocket depth. When the gingival margin is located apical to the cementoenamel junction, the loss of attachment is greater than the probing pocket depth and therefore the distance between the cementoenamel junction and the gingival margin should be added to the probing pocket depth.

Three measurements were made on the buccal aspect and three on the lingual aspect of each tooth – total of six sites per tooth (Mesiobuccal , Midbuccal, Distobuccal, Mesiolingual, Midlingual and Distolingual).From the recorded data Samples that involved number of sites > 30% of examined surfaces[13] were included in the study.

Blood Sample Collection:

5 ml of peripheral blood was obtained by direct venipuncture (21 gauge) using disposable syringe from the antecubital vein of each subject after skin preparation.The samples were immediately transferred into EDTA containing vacutainer tubes. The vacutainer tubes were kept in thermos ice box and transferred immediately to the Department of Genetics, University of Madras, Chennai, where the buffy coat was separated and stored at –20°C until isolation of DNA was done.

DNA Isolation and Purification:

The phenol chloroform method of DNA isolation was used in this study. This frequently used method for DNA isolation removes proteins and other cellular components from nucleic acids, resulting in relatively pure DNA preparations.

I. Principle:

The concept of isolation of DNA is that all the other components of the cell and chromatin are removed using suitable methods to leave behind the DNA. In general the isolation of DNA from mammalian tissues follow four different steps.

1. Lysis of cells with a detergent like sodium dodecyl sulphate (SDS).
2. Digestion of proteins with enzymes (Proteinase – K).
3. Extraction of DNA by phenol chloroform method.
4. Precipitation of DNA with isopropyl alcohol or 100% ethanol.

II. Reagents and their Functions

1. RBC Lysis Buffer

Ammonium chloride – 155mM (8.29g)

EDTA – 0.1mM (1.00g)

NaHCO3 – 12mM (0.034g)

Adjust pH to 7.4 with 1M HCl or NaOH, make upto 1000ml with distilled water. Autoclave and store at room temperature.

The RBC lysis buffer is used to lyse the erythrocytes.

2. SE Buffer/WBC Lysis Buffer

Na2EDTA – 25mM (8.41g)

NaCl – 200mM (11.69g)

Adjust pH to 8.0 with 1M NaOH, make upto 1000ml with distilled water, autoclave and store at room temperature.

3. Proteinase K (10 mg/ml)

Proteinase K - 100 mg

TE - 10 ml

Dissolve 100mg Proteinase K in 10ml TE for 30min at room temperature and store it at -20°C. Proteinase K is the enzyme commonly employed for digestion of proteins. It is a highly active protease purified from the mold *Tritirachium album*. The digestion with proteinase K is usually carried out in presence of EDTA because EDTA inhibits the action of Mg^{2+} ion dependent nucleases which otherwise can digest the DNA.

4. Sodium dodecyl sulphate (SDS) 10%

SDS - 10 gram

Add water to make up to 100 ml, stir on a magnetic stirrer, filter and store at room temperature.

SDS is the commonly used detergent for DNA isolation. It ruptures the cell wall and nuclear membranes to release the contents. Furthermore, it also denatures proteins present in the sample.

5. Phenol (Saturated, pH 8)

Phenol is used to extract the DNA from the solution. In alkaline pH it extracts the DNA to the aqueous phase, which is collected for further purification. This will prevent the contamination of DNA with RNAs. In neutral or acidic pH phenol extracts RNA to aqueous phase. Hence, the pH of phenol is very important for this step. The pH of phenol should be maintained above 7.8 as all eukaryotic RNA with poly-A tails dissolve in alkaline phenol but in the acid range the DNA will partition into organic phase.

6. Phenol: Chloroform : Isoamyl alcohol mixture

To prepare Phenol: Chloroform: Isoamyl alcohol mixture mix 25 parts of Phenol, 24 parts of Chloroform and 1 part of Isoamyl alcohol.

The denaturation of proteins is mainly achieved through the activity of chloroform. It causes surface denaturation of proteins and also helps in removal of fats from the sample. Chloroform also eliminates any traces of phenol as phenol can cause phosphodiester breakage. Mixture of phenol and chloroform is also useful for the removal of protein from nucleic acid samples.

Because of the presence of proteins in the solution, the chance of foaming is more for the solution at the time of phenol: chloroform extraction. The action of isoamyl alcohol is to reduce the foaming and to maintain the stability of layers after centrifugation of deproteinised solution.

7. Isopropyl Alcohol

The action of isopropyl alcohol is to precipitate the DNA leaving RNA and polysaccharides in the solution.

8. 70% Ethanol

Ethanol - 70 ml

Distilled water - 30 ml

It removes residual salt and moisture in the precipitated DNA.

9. Tris-EDTA (TE) Buffer (pH - 8.0)

Tris base - 1.2114 gram

EDTA - 0.0372 gram

Dissolve in 900 ml distilled water and adjust the pH to 8. Make up the volume to 1000 ml. Filter, autoclave and store at 4^{o}C. Ideal buffer to store the DNA.

iii. Procedure

5ml of whole blood was spun at 3500rpm for 20min at 25°C. Buffy coat was removed carefully and transfered to a new 2.0 ml eppendorf tube. 1ml of 1xRBC lysis buffer was added to the buffy coat, vortexed, mixed well and incubated for 15 min at 37°C followed by a spin at 3500rpm for 15 min at room temperature. The supernatant was discarded, the pellet was dislodged and washed 2 or 3 times with 1ml RBC lysis buffer (repeat of step 2) until a half white pellet appears. The pellet was then dislodged by tapping to which 500µl of SE (WBC lysis) buffer, 5µl ProteinaseK (final concentration 50µg/ml) and 25µl (final concentration 0.5%) 10% SDS was added and incubated in water bath at 37°C for overnight or 55°C for 3 hours. Equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) to the lysate was added and intensely mixed well by inverting the tube until it turns to milky white in colour. The samples were spun at 10,000 rpm for 15min at room temperature. The upper aqueous phase alone was carefully collected with the help of wide bore tips without disturbing the other layers and transferred to a new tube. To this aqueous phase 2.5 volumes of chilled absolute ethanol was added and the tubes were inverted gently for several times. The DNA will be visible like a thread and will assume the shape of a cotton ball. The DNA was transferred to an eppendorf tube already containing 1ml of 70% ethanol and spun at 12,000g for 10 min at 4°C. The Ethanol was discarded and the pellet is air-dried in a

sterile place for 3 hours to remove any trace of residual ethanol. Appropriate amount of 1x TE was added according to the size of the pellet, allowed to dissolve and stored at 4°C.

Quality Check & Quantification of DNA

The integrity of the DNA was assessed by running it in 0.7% Agarose gel. Further the quantification and quality check of DNA was performed by subjecting the DNA to spectrophotometry.

I. Principle

The concept of quality check of DNA is to find out the purity of the extracted DNA. The extracted DNA may contain impurities like phenol, proteins and others. The integrity of the DNA is checked by agarose gel electrophoresis.

When the DNA is mixed with loading dye and run electrophoretically on 0.7% agarose gel in TAE buffer, the good high molecular weight DNA will appear as sharp band without smearing.

II. Reagents

1) TAE buffer (10x)

Tris base - 48.4 gram

Glacial acetic acid - 11.42 ml

0.5 M EDTA (pH 8.0) - 20 ml

Distilled water to make up to 1000 ml

Autoclave and store at room temperature.

2. Gel loading dye – Type III (6x)

Bromophenol blue	-	0.25% (w/v)
Xylene cyanol FF	-	0.25% (w/v)
Glycerol in water	-	30% (v/v)

Stir well and store at $4^{o}C$.

3. Ethidium bromide

Ethidium bromide	-	10 mg
Distilled water	-	1 ml

Mix well to ensure that the dye has dissolved completely. Wrap the tube in aluminum foil and store at room temperature.

III. Procedure for Agarose Gel Electrophoresis

1. 0.7% Agarose Gel preparation

- 0.7 gram of agarose weighed and transferred into a 250 ml conical flask.
- 100 ml of 0.5x TAE buffer was added to it, stirred well and melted on a magnetic stirrer cum hot plate until the agarose dissolves completely.
- The appropriate sized gel tray and comb was washed and wiped with 70% Ethanol. The gel tray was placed inside the casting unit. The comb was placed on the gel tray and left on an even surface.
- After the agarose cools down to hand bearing temperature, 5 µl of ethidium bromide was added and mixed well. It was poured on the gel tray and allowed to polymerize. After polymerization the comb is removed gently.

2. Preparation of sample and loading

- The gel tray was removed from the casting unit and the tray placed in the electrophoresis tank.
- 0.5x TAE buffer was poured into the tank until the gel gets immersed.
- 2 µl of each DNA sample was taken and mixed with 2µl of 6x loading dye and 8ml of sterile double distilled water.
- The DNA samples were loaded into the wells.
- The electrodes were connected.
- The power was switched ON, set at 100 V.
- As the DNA is negatively charged, it will migrate towards the anode.

3. Visualizing the DNA

- When the bromophenol blue dye was in the middle of the gel, the power was switched OFF.
- The gel was taken to the transilluminator and observed under UV and documented.
- The good high molecular weight DNA will appear as sharp band without smearing.

IV. Procedure for Spectrophotometry

The nucleic acid sample was analysed at 260nm and 280nm by using Nanodrop Spectrophotometer (Thermo scientific, Germany). The concentration and purity of the sample was analysed using the following formula,

Concentration of DNA:

Concentration of double stranded DNA sample (µg/µl) = A260 x 50

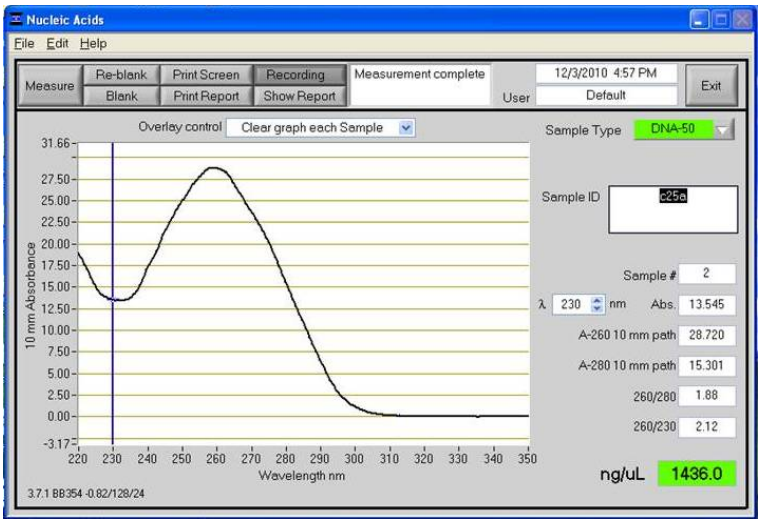
Purity of DNA:

Pure DNA = A260 /A280 ≥ 1.8

< 1.8 indicates protein and phenol contamination

>2.0 indicates the possible contamination with RNA

Figure 1: Nanodrop spectrophotometer window of a representative sample showing the absorption spectra of DNA (peaks at 260nm)



DNA Dilution:

After confirmation for the presence of genomic DNA in the sample and quantification, the sample has to be diluted with autoclaved sterile double distilled water, to make it amenable to be used in polymerase chain reaction. The amount of DNA needed for PCR is 50-100 ng of DNA for a 20 µl reaction mixture.

Once a working DNA sample has been prepared, it was run in 1% agarose gel electrophoresis, observed under UV and documented.

POLYMERASE CHAIN REACTION

I. Principle

PCR entails enzymatic amplification of specific DNA sequences using two oligonucleotide primers that flank the DNA segment to be amplified. The rapid production of large quantities of a specific DNA sequence took a leap forward with the development of the PCR. The PCR requires two nucleotide oligomers (Primers) that hybridize to the complimentary DNA strands in a region of interest. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA.

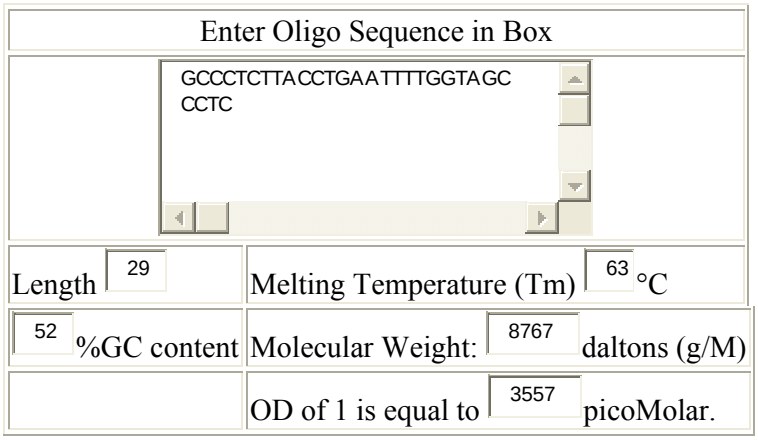
II. Reagents

1. 10x PCR buffer
2. 25 mM $MgCl_2$
3. 2.5mM dNTP Mix
4. **Forward Primer (Sigma Aldrich, USA)**

The sequence is

5'- GCCCTCTTACCTGAATTTTGGTAGCCCTC-3'

Figure 2: Oligo Calculator – A bioinformatic tool that analyses the melting temperature and other details of Primer sequences: Shown below is the Forward Primer

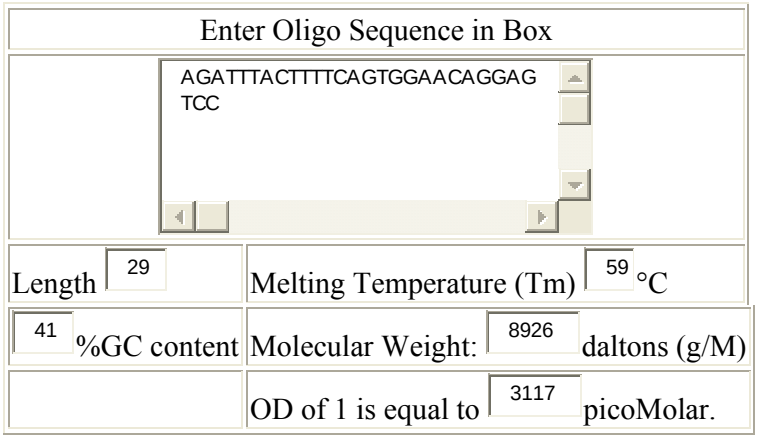


5).Reverse primer (Sigma Aldrich, USA)

The sequence is

5' - AGATTTACTTTTCAGTGGAACAGGAGTCC - 3'

Figure 3: Oligo Calculator – A bioinformatic tool that analyses the melting temperature and other details of Primer sequences: Shown below is the Reverse Primer



Preparation of the primer:

The primer is obtained as lyophilized powder and is reconstituted in appropriate volume of sterile triple glass distilled water to a concentration of 100μM. A working stock of 2micro Mole primer is prepared and stored at -20°C

The Primers were adapted from Folwaczny M *et al*., 2005[21].

Amplicon size: 171bp

Figure 4: In-Silico PCR showing the region of DNA to which the primer binds and amplifies (UCSC Genome Bioinformatics Database)

UCSC In-Silico PCR

>chr11:112035360-112035530 171bp
GCCCTCTTACCTGAATTTTGGTATCCCTC
AGATTTACTTTTCAGTGGAACAGGAGTCC
GCCCTCTTACCTGAATTTTGGTATCCCTCtccccaagcttactttctgtt
gcagaaagtgtaaaaattattaaataaaattctaatgatggtatccgtgt
ggcttgcatctgatacagcagataaagaagttttatgaaaatGGACTCCT
GTTCCACTGAAAAGTAAATCT

6)Taq DNA polymerase (Applied Biosystems, USA)

Taq DNA polymerase is a highly thermostable DNA polymerase of a thermophilic bacterium *Thermus aquaticus*. Taq DNA polymerase catalyzes 5'→3' synthesis of DNA.

7. PROCEDURE

- 2µl of genomic DNA (100ngµl) is pipetted directly to the bottom of the labelled PCR tubes.
- Preparation of master mix: A master mix of all the components of PCR except the genomic DNA was prepared as follows:

Stock Concentration	**Contents**	**Quantity**	**Final Concentration (20µl)**
10X	PCR Buffer	2.0µl	1X
25mM	MgCl2	2.0µl	2.5mM
2.5mM	dNTP mix	0.8µl	50µM
2µM	Forward Primer	0.8µl	80nM
2µM	Reverse Primer	0.8µl	80nM
5U/µl	Taq DNA Pol	0.1µl	0.25U/10µl
	Distilled water	11.5µl	
	Total	**18.0 µl**	
100ng/µl	Genomic DNA	2.0µl	100ng/10µl

Thermal Cycle Protocol

- The PCR mix was added into the sterile 0.2ml PCR tube and the following thermal cycle conditions were programmed in GeneAMP® PCR System 9700-Applied Biosystems, USA.

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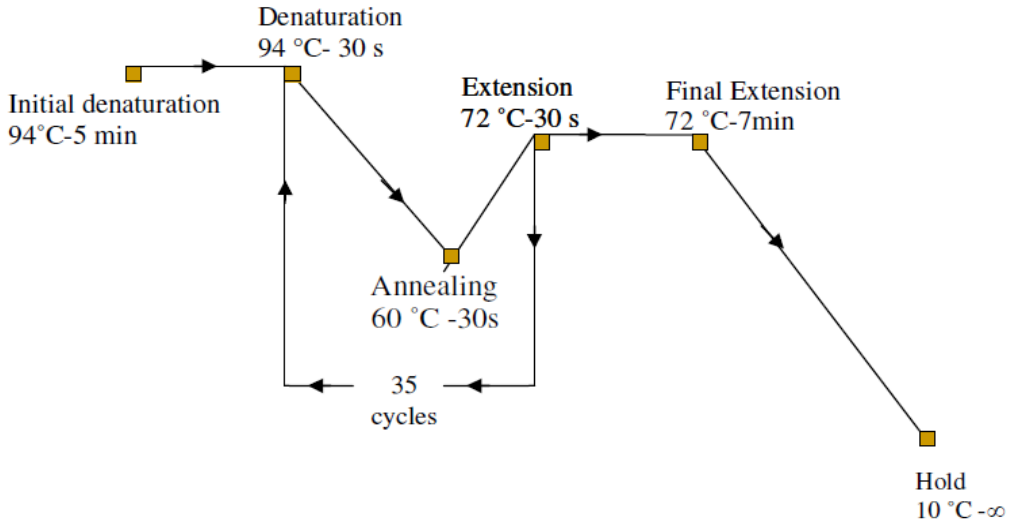
Figure 5: PCR thermal cycle. Figure depicting the temperature condition and cycles during different phases of PCR.



After the programme is over, the samples were tested for amplification by 2% agarose gel electrophoresis and visualized with ethidium bromide.

The correct polymerase chain reaction product is 171 bp long.

Restriction Fragment Length Polymorphism (RFLP)

I. Principle

In this method, DNA sequence variation is identified by amplification of the region using polymerase chain reaction followed by digestion of the amplified product with a restriction endonuclease known to be capable of distinguishing the polymorphic patterns. The restriction fragments vary in size and can be revealed as different bands on gel electrophoresis.

II. Reagents

1. **10x Nuclear Extract Buffer 4(NE Buffer)**

   50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9.

2. **Bovine serum Albumin (BSA)**

3. **MseI (New England Biolabs Inc., Beverly, MA, USA)**

4. This is the restriction endonuclease can digest the AA but not the AC or CC genotype.

Recognition Site:

iii. Restriction Endonuclease Digestion Procedure

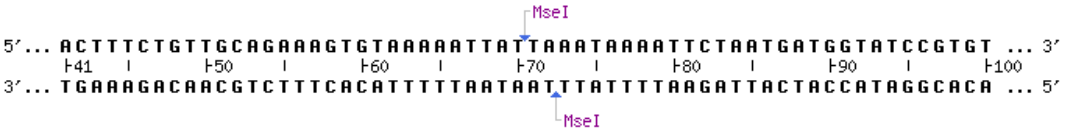
			One Sample
1.	PCR product	-	10 µl
2.	10x NE buffer	-	2 µl
3.	10x Bovine serum albumin	-	2 µl
4.	MseI (10 units/ml)	-	0.1 µl
5.	Triple glass distilled water	-	5.9 µl
	Total	-	20 µl

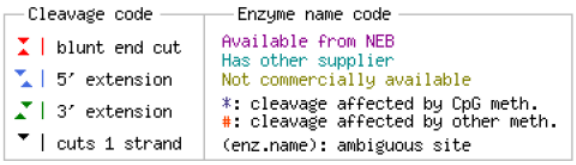
- Pipette PCR product separately to each labelled tube and prepare master mix of the remaining items for the required number of samples.
- Dispense 10 µl of master mix into each tube containing PCR product.
- Spin the tubes briefly to collect the contents at the bottom and incubate at 37^{o}C overnight in a water bath.
- Inactivate the enzyme by incubating at 65^{o}C for 20 mins.
- Resolve the fragments by electrophoresis using 2% agarose and visualise in a UV transilluminator after staining with ethidium bromide.

Figure 6: In-Silico Analysis of RFLP:

Wild type Sequence digested with: *MseI*

Figure 7: The DNA sequence of the site which is restricted by *MseI*

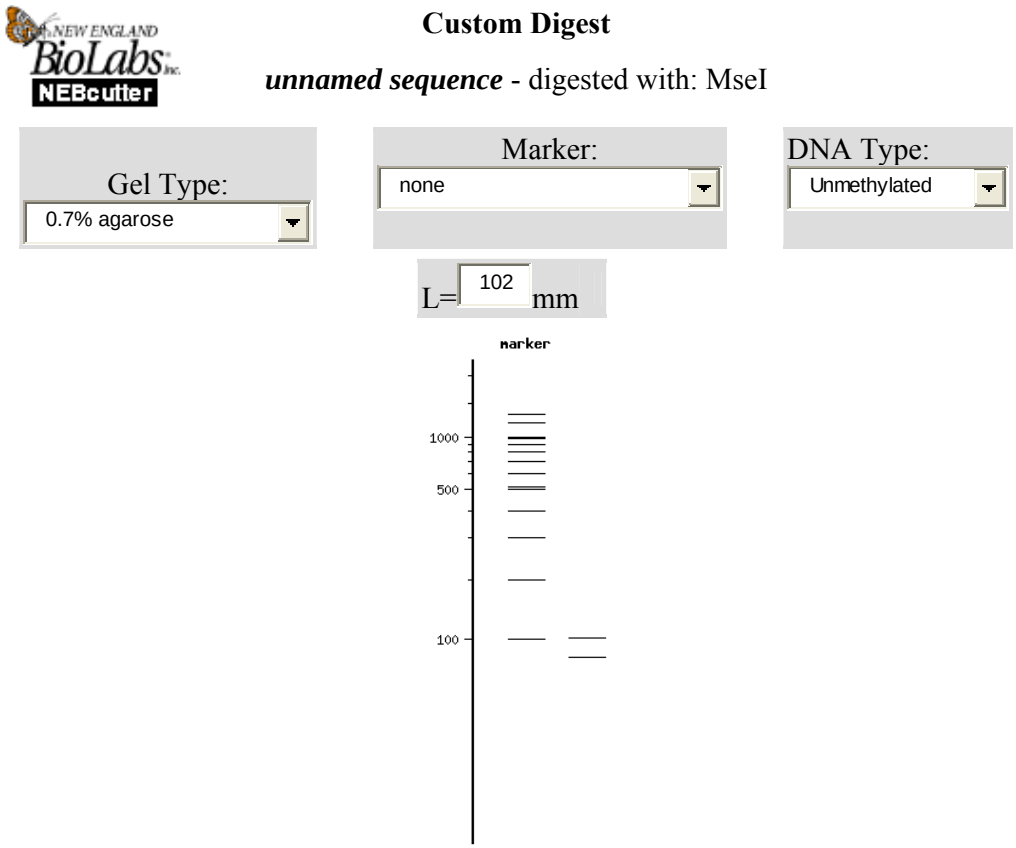




Fragment sizes when restricted

#	Ends	Coordinates	Length (bp)
1	(LeftEnd)-MseI	1-70	70
2	MseI-(RightEnd)	71-171	101

Figure 8: In-Silico Gel showing the restriction fragments when run in 2% Gel



The virtual gel was generated by New England Biolabs Virtual Gel Bioinformatics tool

Source: NEB cutter V.2

ARMAMENTARIUM

For Periodontal Examination and Blood Sampling

- Mouth mirror
- Williams periodontal probe
- Tweezer
- Sterile gauze
- Mask
- Head cap
- Gloves
- 5ml disposable syringe
- EDTA containing vaccutainer

For DNA Isolation

- Centrifuge tubes – 15ml
- Centrifuge machine
- Wide bore Pasteur pipettes
- Micropipette
- TKM 1 (Low salt buffer)
- Igepal CA – 630
- TKM2
- (10 % SDS)
- 6M sodium chloride
- Tris-EDTA buffer (TE buffer)

- Iso propyl alcohol
- 70% ethanol
- Tris EDTA buffer
- Centrifuge – Remi
- Vortexer
- Water bath incubator
- Refrigerated centrifuge

For Electrophoresis

- Agarose
- 10x TAE buffer
- Microwave oven
- Electrophoresis tank with power pack
- Electrophoresis gel tray
- Gel loading loading dye
- Micropipette
- Ethidium bromide
- U – V light transilluminator

For PCR

- 0.5 ml eppendorf tube
- Taq polymerase
- Forward primer
- Reverse primer

- dNTP
- $Mgcl_2$
- KCl buffer
- Template DNA
- Thermalcycler
- Nanocentrifuge
- 100 bp DNA ladder
- TGDW

For RFLP

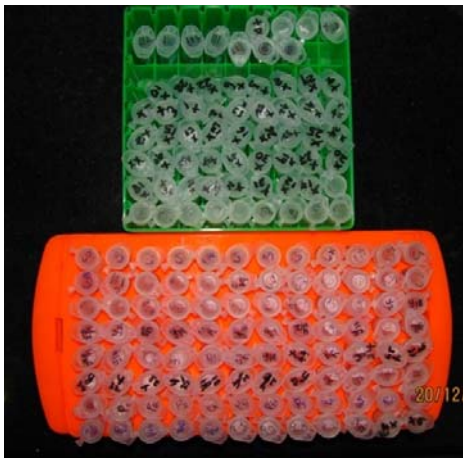
- PCR product
- Restriction Endonuclease enzyme (MseI)
- Buffer
- TGDW
- Water Bath Incubator

Photograph 1: Armamentarium for Clinical Examination

Photograph 2: Armamentarium for blood sample collection and transportation

Photograph 3: An illustration for blood sample collection

Photograph 4: Collected samples



Photograph 5: Control Group – HealthyPeriodontium

Photograph 6: Study group-Chronic Periodontitis

Photograph 7: Centrifuge

Photograph 8: Refrigerated Centrifuge

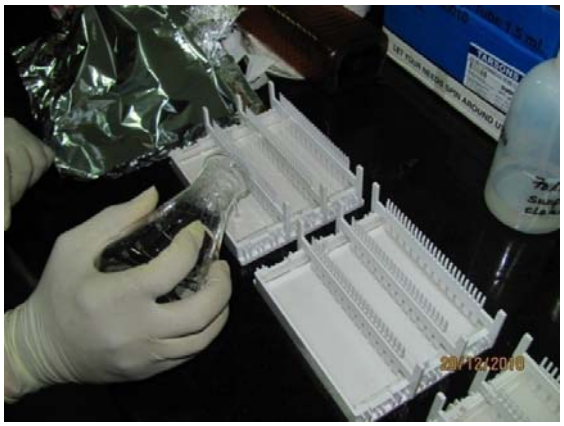
Photograph 9: Water Bath

Photograph 10: Vortex Mixer

Photograph 11: Reagents for DNA Isolation and Purification

Photograph 12: Isolated DNA

Photograph 13: Gel tray and Gel Comb with Agarose



Photograph 14: Electrophoresis Tank

Photograph 15: PCR hood

Photograph 16: UV Transilluminator

Photograph 17: Microcentrifuge

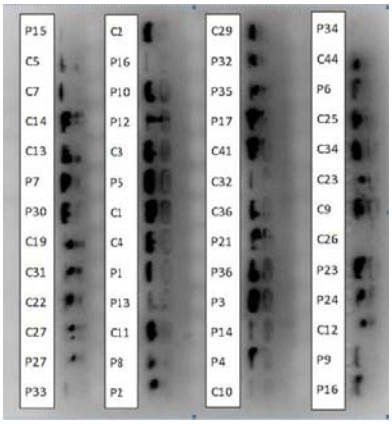
Photograph 18: PCR Thermal Cycler

Photograph 19:
Centrifuge Tubes, Pasteur Pipettes, Eppendorf and PCR Tubes

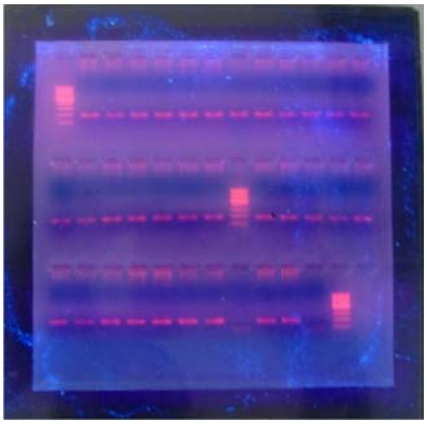
Photograph 20: Micropipettes

Photograph 21: Primers and Taq DNA polymerase
Restriction enzyme MseI, NE buffer and BSA

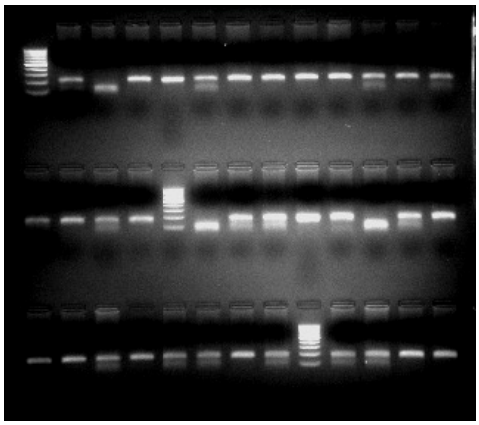
Photograph 22: Electrophoresis Results of Genomic DNA – Stock –Sample



Photograph 23: Electrophoresis Results of Polymerase Chain-Sample illustration



Photograph 24: Electrophoresis Results of RFLP-Sample illustration



STATISTICAL ANALYSIS

Around 200 samples of Tamil speaking Dravidians were selected from the out patient wing of Department of Periodontology, Tamilnadu Government Dental College,Chennai. Based on the AAP classification system and percentage of number of involved sites. The groups were divided into control and study group. 181 samples were included (n=100 for control group and n=81 for study group) for PCR and RFLP analysis after checking the DNA quality and quantity based on UV spectrometric analysis. The study design was devised based on case control model. Those 181 RFLP digested product was categorized into three genotypes namely CA, AA and CC for control and study group separately. Age, gender, gingival bleeding index and plaque index were included in master chart for control and study group. Further samples with smoking habit in the study group are noted separately.

It is supposed that age, gender and smoking could act as confounders influencing disease outcome[37]. These factors are need to be corrected or adjusted to really associate the effect of single nucleotide polymorphism in IL-18 coding region. Hence a multivariate analysis model was adopted to adjust the covariates assuming that C or A allele act either in productive or destructive manner. But further statistical analysis revealed no significant difference in distribution of alleles (C or A) or genotypes (CC, CA and AA) among the total study population (includes study and control group).

As there was no association established between the SNP in IL-18 coding region of our interest. It is unnecessary to adjust the effect of covariates on disease

outcome among total study population. So multivariate analysis model was dropped out. Hence usual statistical tools were used in this study

The statistical package SPSS PC + (Statistical Package for Social Science, Version 18) was used for statistical analysis.

Analysis of variance (ANOVA) followed by Tukey – HSD test was the technique used in this study to compare the mean values between control, study group A and B. ANOVA is used to test equality of means, when more than two populations are considered.

A set of N = Cr observations classified in one direction may be represented as follows:

$$\text{Mean} = \frac{X_{1r} \quad X_{2r} \quad X_{cr}}{\overline{X}_1 \quad \overline{X}_2 \quad \overline{X}_c}$$

$$\overline{\overline{X}} = \frac{\overline{X}_1 + \overline{X}_2 + \ldots\ldots \overline{X}_c}{C}$$

Then

`Between column' sum of squares : SSC = $\sum_j \left(X_j - \overline{\overline{X}}\right)^2$

`Within column' sum of squares : SSE = $\sum_j \sum_i \left(X_{ij} - \overline{X_j}\right)^2$

Total sum of squares : SST = $\sum\sum \left(X_j - \overline{\overline{X}}j\right)^2$

SST = SSC + SSE

Anova Table

Source of Variation	Sum of squares	Degrees of freedom	Mean sum of squares	Variance ratio
Between columns	SSC	C-1	$MSC = \frac{SSC}{C - 1}$	$F = \frac{MSC}{MSE}$
Within Columns	SSE	N-C	$MSE = \frac{SSE}{N - 1}$	$\overline{MSE} = \frac{\overline{MSE}}{MSC}$
Total	SST	N-1		

CHI-SQUARE TEST

Chi-square test with Pearson's Correlation Coefficient was employed to compare the proportion between cases and controls. The formula used as

$$X^2 = \frac{\Sigma(O-E)^2}{E}$$

where O is the observed frequency and E is the expected frequency in a cell.

Pearson's correlation coefficient may be calculated as

$$r = \frac{\frac{1}{n}\sum_{1}^{n}\left(x-\bar{x}\right)\left(y-\bar{y}\right)}{\frac{1}{n}\sum_{1}^{n}\left(x-\bar{x}\right)^2 \frac{1}{n}\sum_{1}^{n}\left(y-\bar{y}\right)^2}$$

Regression is calculated as

$$y = a_0 + a_1 x + \varepsilon$$

Where a_0 = Intercept

a_1 = Slope

In the present study P value < 0.05 was considered as the level of significance.

ONEWAY ANOVA

In a one-way ANOVA, the F statistic tests whether the treatment effects are all equal, i.e. that there are no differences among the means of the J groups. A significant F value indicates that there are differences in the means, but it does not tell you where those differences are, e.g. group 1's mean might be different than group 2's mean but not different from group 3's mean.

To isolate where the differences are, you could do a series of pairwise T-tests. The problem with this is that the significance levels can be misleading. For example, if you have 7 groups, there will be 21 pairwise comparisons of means; if using the .05 level of significance, you would expect at least one statistically significant difference even if no differences exist.

Therefore, various methods have been developed for doing multiple comparisons of group means. In SPSS, one way to accomplish this is via the use of the /POSTHOC parameter on the One-way command. We'll present the SPSS output and then explain what the different parts mean.

MEAN DIFFERENCE

- This column gives the difference in the means of the 2 groups
- STANDARD ERROR.
- In a One-way Anova, the standard error of the difference between the two means of groups i and j is

SIGNIFICANCE:

This column gives you the significance of the difference under the multiple comparison method being used. To understand this, we need explain following method being used and what their rationale is.

When the decision from the One-Way Analysis of Variance is to reject the null hypothesis, it means that at least one of the means isn't the same as the other means. What we need is a way to figure out where the differences lie, not just that there is a difference.

This is where the Tukey tests come into play. They will help us analyze pairs of means to see if there is a difference much like the difference of two means covered earlier.

The formulas refer to mean i and mean j. The values of i and j vary, and the total number of tests will be equal to a combination of k objects, 2 at a time C(k,2), where k is the number of samples.

INDEPENDENT t TEST

The independent t-test, as we have already mentioned is used when we wish to compare the statistical significance of a possible difference between the means of two groups on some independent variable and the two groups are **independent** of one another.

The formula for the independent t-test is

$$t = \frac{X_1 - X_2}{\sqrt{\left(\frac{SS_1 + SS_2}{n_1 + n_2 - 2}\right)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}},$$

where

X_1 is the mean for group 1,

X_2 is the mean for group 2,

SS_1 is the **sum of squares** for group 1,

SS_2 is the **sum of squares** for group 2,

$\mathbf{n_1}$ is the number of subjects in group 1, and

$\mathbf{n_2}$ is the number of subjects in group 2.

The sum of squares is a new way of looking at variance. It gives us an indication of how spread out the scores in a sample are. The t-value we are finding is the difference between the two means divided by their sum of squares and taking the degrees of freedom into consideration.

$$SS_1 = \sum X_1^2 - \frac{\left(\sum X_1\right)^2}{n_1}$$

and

$$SS_2 = \sum X_2^2 - \frac{\left(\sum X_2\right)^2}{n_2}$$

We can see that each sum of squares is the sum of the squared scores in the sample minus the sum of the scores quantity squared divided by the size of the sample (n).

So to calculate the independent-t value we need to know:

1. The mean for sample or group 1
2. The mean for sample or group 2
3. The summation X and summation X squared for group 1
4. The summation X and summation X squared for group 2
5. The sample size for group 1 (n_1)
6. The sample size for group 2 (n_2)

We also need to know the degrees of freedom for the independent t-test which is:

$$df = n_1 + n_2 - 2.$$

RESULTS

Tables -1. Shows percentage of control and study population among total sample size we infer that the percentage of control population among total sample size, 55.2% and percentage of study population among total sample size, 44.8%

Tables -2. Shows the percentage of different genotypes among total sample size we infer that the percentage of AA genotypes among total sample size, 7.7% percentage of CA genotypes among total sample size, 42.0% and percentage of CC genotypes among total sample size 50.3%.

Tables-3 Shows the Mean, SD values of plaque index and gingival bleeding index for different genotypes among control and study group the inference as follows

A. Plaque Index for Control group

i. In AA genotype the Mean and SD for plaque index, 0.69% and 0.20% respectively.

ii. In CA genotype the Mean and SD for plaque index, 0.76% and 0.18% respectively.

iii. In CC genotype the Mean and SD for plaque index, 0.81% and 0.17% respectively.

B. Gingival Bleeding Index for control group

i. In AA genotype the Mean and SD for bleeding index, 2.89% and 1.79% respectively.

ii. In CA genotype the Mean and SD for bleeding index, 2.86% and 2.31% respectively.

iii. In CC genotype the Mean and SD for bleeding index, 2.87% and 1.74% respectively.

A. Plaque Index for study group

i. In AA genotype the Mean and SD for plaque index, 0.40% and 0.15% respectively.

ii. In CA genotype the Mean and SD for plaque index, 0.41% and 0.07% respectively.

iii. In CC genotype the Mean and SD for plaque index, 0.38% and 0.06% respectively.

B. Gingival Bleeding Index for Study group

i. In AA genotype the Mean and SD for bleeding index, 10.03% and 3.79% respectively.

ii. In CA genotype the Mean and SD for bleeding index, 5.96% and 1.05% respectively.

iii. In CC genotype the Mean and SD for bleeding index, 5.37% and 0.83% respectively.

Table-4 Shows the difference between control and study group for PlI, GBI among all the genotypes we infer that the difference between control and study group for PlI, GBI among all the genotypes is significant as the p value is less than 0.05.

Table-5 Shows the one way analysis from which we infer that

A. For control group

i. Mean and SD for GBI in genotype AA is 2.89 and 1.79

ii. Mean and SD for GBI in genotype CA is 2.86 and 2.31

iii. Mean and SD for GBI in genotype CC is 2.87 and 1.74

B. For study group

i. Mean and SD for GBI in genotype AA is 79.26 and 10.03

ii. Mean and SD for GBI in genotype CA is 79.13 and 5.96

iii. Mean and SD for GBI in genotype CC is 77.49 and 5.37

Table:6 Show the difference for GBI, within the samples of all three genotypes and between the three genotypes separately for control and study group we infer that the difference for GBI, within the samples of all three genotypes and between the samples of three genotype for control and study group is insignificant as the p value is greater than 0.05.

Tables: 7 shows the distribution of A and C alleles among control and study group we infer that.

i. Total no of A allele in control group 58

ii. Total no of A allele in study group 46

iii. Total no of C allele in control group 142

iv. Total no of C allele in study group 116

Tables: 8 Shows Chi-square test for genotype distribution which is insignificant between control study group.

Tables: 9 Shows the Chi-square test for allele frequency we infer that the table shows percentage distribution and allele frequency for A and C allele among control and study group is insignificant as the p values is greater than 0.05. This implies neither of the allele A and C is predominately distributed among control and study group.

Tables: 10 Shows the Mean and SD of all the genotypes for samples in study group with and without smoking habit we may infer as follows

A. In samples with smoking habit

i. The Mean value for PlI and GBI in CA genotypes are 1.80 and 76.33 respectively

ii. The Mean value for PlI and GBI in CC genotypes are 1.59 and 76.22 respectively

iii. No AA genotyped samples were found in samples with smoking habit

B. In samples without smoking habit

i. The Mean value for PlI and GBI in AA genotypes are 1.69 and 79.26 respectively

ii. The Mean value for PlI and GBI in CA genotypes are 1.67 and 79.32 respectively

iii. The Mean value for PlI and GBI in CC genotypes are 1.64 and 78.12 respectively

Table 11 Shows the significance of difference for PlI and GBI among all the genotypes of study group that involves samples with and without smoking habit. We infer that there is no significant difference for PlI and GBI among all the genotypes of study group that involves samples with and without smoking habit

Photograph 25-29 shows the electrophoresis result of PCR product 171bp. Lane 1,20,35 are positive control – standard 100 bp DNA ladder.

Photograph 30-34 shows electrophoresis result of RFLP product for all genotypes CA,CC AND AA in control and study group we infer that

- A thin uncut band along with restricted 171 bp as 101 and 70 –for CA genotype
- A thick uncut band for 171bp -for CC genotype
- Restricted site for both the alleles for 171 bp as 101bp and 71bp for –AA genotype.

Tables 1: Percentage of control and study population among total sample size

	Group	
	N	%
Control	100	55.2
Study	81	44.8
Total	181	100.0

Tables 2: Percentage of different genotypes among total sample size

	N	%
AA	14	7.7
CA	76	42.0
CC	91	50.3
Total	181	100.0

Tables 3: Mean, SD values of plaque index and GBI for different genotypes among control and study group.

		Group							
		Control				Study			
Genotype		Mean	SD	SE	N	Mean	SD	SE	N
AA	PlI	.69	.20	.08	7	1.69	.40	.15	7
	GBI	2.89	1.79	.68	7	79.26	10.03	3.79	7
CA	PlI	.76	.18	.03	44	1.67	.41	.07	32
	GBI	2.86	2.31	.35	44	79.13	5.96	1.05	32
CC	PlI	.81	.17	.02	49	1.63	.38	.06	42
	GBI	2.87	1.74	.25	49	77.49	5.37	.83	42

Table 4: Difference between control and study group for PlI,GBI among all the genotypes.

T-Test

Independent Samples Test						
		Levene's Test for Equality of Variances		t-test for Equality of Means		
Genotype		F	Sig.	t	df	Sig. (2-tailed)
AA	PlI	15.150	.002	-5.970	8.875	.000
	GBI	4.372	.058	-19.827	12	.000
AC	PlI	36.254	.000	-11.698	40.047	.000
	GBI	26.743	.000	-68.697	37.809	.000
CC	PlI	31.593	.000	-12.945	55.616	.000
	GBI	28.616	.000	-86.220	48.335	.000

Table 5: One way analysis

Group				Mean	SD	SE	N
Control	Gene	AA	GBI	2.89	1.79	.68	7
		AC	GBI	2.86	2.31	.35	44
		CC	GBI	2.87	1.74	.25	49
Study	Gene	AA	GBI	79.26	10.03	3.79	7
		AC	GBI	79.13	5.96	1.05	32
		CC	GBI	77.49	5.37	.83	42

Table 6: Difference for GBI, within the samples of all three genotypes and between the three genotypes separately for control and study group

ANOVA

Group			Sum of Squares	df	Mean Square	F	Sig.
Control	GBI	Between Groups	.005	2	.002	.001	.999
		Within Groups	393.402	97	4.056		
		Total	393.407	99			
Study	GBI	Between Groups	56.324	2	28.162	.760	.471
		Within Groups	2890.285	78	37.055		
		Total	2946.609	80			

Tables 7: Distribution of A and C alleles among control and study group.

	Allele	
	A	C
Control	58	142
Study	46	116

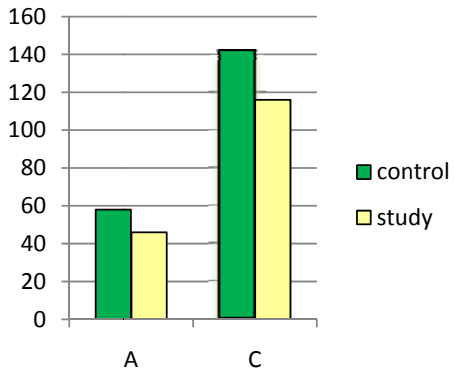


Figure 9: Bar Diagram

Tables 8: Chi-square test for genotype distribution

	Control (%)	Study Group (%)	x2	p value
AA	7 (7)	7 (8.54)	0.332	0.839
AC	49 (49)	41 (51.22)		
CC	44 (44)	33 (40.24)		

Tables 9: Chi-square test for allele frequency

	Control (%)	Study group (%)	x2	p value
A	58 (29)	46 (39.7)	0.005	0.932
C	142 (71)	116 (71.34)		

Tables 10: Mean and SD of all the genotypes for samples in study group with and without smoking habit.

	Group							
	Study Group							
	Smoking Habit							
	Yes				No			
	Plaque Index		Bleeding Index		Plaque Index		Bleeding Index	
Gene	Mean	N	Mean	N	Mean	N	Mean	N
AA	.		.		1.69	7	79.26	7
CA	1.80	2	76.33	2	1.67	30	79.32	30
CC	1.59	14	76.22	14	1.64	28	78.12	28

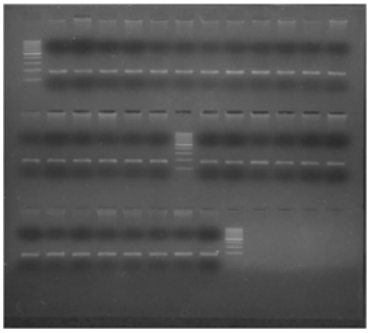
Table 11: Significance of difference for PlI and GBI among all the genotypes of study group that involves samples with and without smoking habit.

T-Test

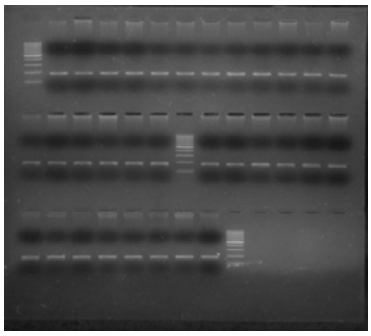
Independent Samples Test(a)						
		Levene's Test for Equality of Variances		t-test for Equality of Means		
Gene		F	Sig.	T	df	Sig. (2-tailed)
CA	PlI	1.177	.287	.456	30	.652
	GBI	1.139	.294	-.680	30	.502
CC	PlI	1.450	.236	-.372	40	.712
	GBI	1.974	.168	-1.084	40	.285

PHOTOGRAPH FOR PCR

Photograph 25



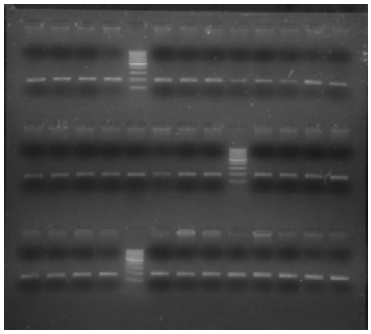
Photograph 26



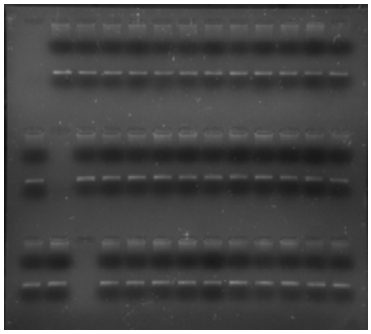
Amplicon size 171bp

100bp ladder

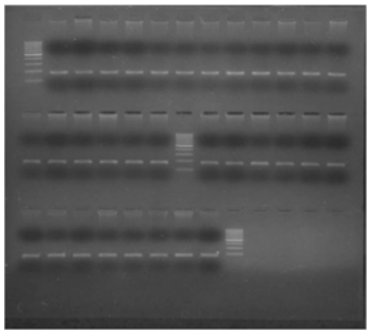
Photograph 27



Photograph 28

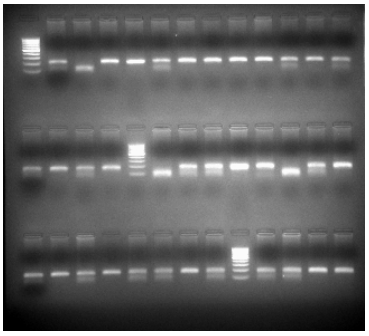


Photograph 29

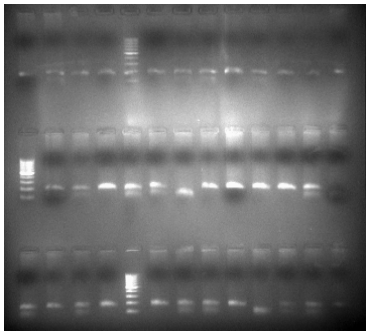


PHOTOGRAPH FOR RFLP

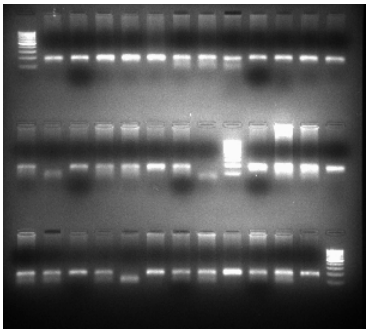
Photograph 30



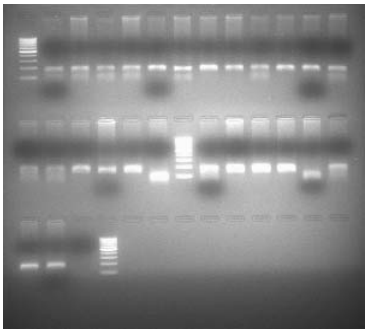
Photograph 31



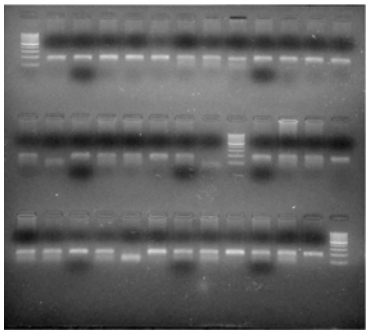
Photograph 32



Photograph 33



Photograph 34



DISCUSSION

Unraveling the pathophysiology of destructive periodontal disease comprises one of the major challenges in periodontology[72]. Currently, genetic factors causing imbalances in pro-and anti-inflammatory cytokine production along with an ineffective immune response against bacterial invasion were thought to increase the susceptibility for periodontitis[6].

Among the different cytokines that are typically expressed in periodontal lesions specifically the level of IFN-γ was suggested to be positively correlated with the progression of the disease[87]. A considerably higher level of IFN-gamma was found in aggressive periodontitis patients and in active periodontal pockets suggesting a destructive role for IFN- γ [24]. Moreover, studies in rodents reported a significantly decreased bone loss for mice lacking the cytokine IFN-gamma suggesting an important role for IFN- γ in periodontitis associated tissue destruction[6,33]. IL-18 plays an important role in the regulation of IFN- γ production. IL-18 protein expression is regulated by the IL-18 promoter gene[85]. Two single nucleotide polymorphisms within the promoter region have recently been described that were suggested to impair IL-18 expression. At position -607 the change from cytosine to adenine disrupts a binding site for CREB (cyclic AMP-responsive element-binding protein) and at position -137 the change from guanine to cytosine affects the H4TF-1 (human histone H4 gene-specific transcription factor-1) binding site[82].

Upon stimulation low promoter activity was observed for A and C alleles at positions -607 and -137, respectively[28]. Consistently, it was shown that the

polymorphisms that have been investigated herein reach significant impact on the pathophysiology of various inflammatory and/or autoaggressive conditions, e.g. rheumatoid arthritis[46], sarcoidosis[82], and type 1 diabetes[34]. However, the functional influence of the polymorphisms investigated herein still remains to be proved.

The frequency of the alleles and genotypes for the IL-18 gene polymorphisms observed herein is consistent with previous data from other study groups[46,84]. The present study revealed no association between any of the IL-18 gene polymorphisms and the susceptibility for periodontitis. Both the frequencies for the alleles and the distribution of genotypes were almost equal among the two study groups. It is clear from studies that the IL-18 molecule shares structural homology with the IL-1 family[16,26]. This holds the value to be related to a recent study on association of Single Nucleotide Gene Polymorphism at Interleukin-1b +3954, -511, and -31 in Chronic Periodontitis and Aggressive Periodontitis in Dravidian Ethnicity found association for In the Malayalam-speaking Dravidian population[2]. In that study allele C of IL-1b +3954 appeared to be an important risk factor for chronic periodontitis. But in our study we find no association for C-A snp at position -607 promotor region of intrleukin 18 coding region considering A allele as risk allele based on the Cloning and mutation analysis of the human IL-18 promoter region[28]. As we didn't find SNP distribution for other five position of IL-18, we may not be able to conclude that IL-18 promotor and coding region polymorphisms are insignificant among periodontontitis patients.

Our study is consistent with earlier findings of a previous study[21] in which the distribution of genotypes for the IL-18 gene polymorphism at position-607 as

insignificant whose p value is 0.854. The p value of our study is 0.839, a comparable integer to the p value of above mentioned study. Further we found the percentage distribution of allele frequency for A and C allele among control and study group is statistically insignificant as the p values is greater than 0.05 (p value 0.932). This implies neither of the allele A and C is predominately distributed among control and study group.

Hence we infer that the distribution of genotypes and allele frequency for the IL-18 gene polymorphism at position-607 was not significantly different among periodontitis patients if compared with healthy control subjects in total sample of Tamil speaking Dravidians. As we didn't find the association for any allele in periodontal health and disease, multivariate analysis model to control the confounders was not developed.

Considering the complex and redundant regulation of cytokine cascades that occurs in inflammation, it is reasonable to anticipate that polymorphisms at multiple genes may influence the qualitative and quantitative aspects of periodontal inflammation. Therefore, to determine the exact direct and epistatic (interaction among multiple alleles) involvement of the IL-18 gene in periodontitis, further studies that simultaneously examine the distribution and dynamics of genetic variation at many loci on the IL-18 gene are essential. Studies that combine many genetic variants, even with moderate effects, may substantially improve our ability to predict periodontitis risks and the clinical use of genetic research.

SUMMARY AND CONCLUSION

About 200 patients in the age group of 30- 70 years who attended the out patient wing of Department of Periodontology, Tamilnadu Government Dental College, Chennai during March to December of 2010 participated in the study . The patients were divided into two groups, 81 subjects with generalized chronic periodontitis and 100 periodontally healthy subjects included as study and control group respectively. All were Tamil speaking Dravidians.

Plaque index, gingival bleeding index, probing depth and clinical attachment level. (Based on AAP-1999 classification), were used to categorize the patients to Control Group, Study Groups (Generalised Chronic Periodontitis). Generalised chronic periodontitis cases were selected based on the percentage of involved no of sites[13].

Five ml of blood was drawn from antecubital vein and DNA was isolated by the Non-enzymatic method. The concentration of the isolated DNA was adjusted suitably and PCR was carried out to amplify the IL- 18 promotor region at -607 position. Allelic variants at -607 position were identified by restriction fragment length polymorphism (RFLP) using MseI enzyme. Data thus collected were analysed statistically.

We found the percentage distribution of allele frequency for A and C allele among control and study group is statistically insignificant as the p values is greater than 0.05 (p value 0.932). This implies neither of the allele A and C is predominately distributed among control and study group. The study results are in consistent with

earlier findings of a previous study[21] in which the distribution of genotypes for the IL-18 gene polymorphism at position-607 as insignificant whose p value is 0.854. The p value of our study is 0.839 is comparable to the p value of above mentioned study.

Hence the present findings indicate that the susceptibility for periodontitis is not influenced by the SNP at -607of IL 18 gene that have been investigated in this study and we find no association between them. Herein the distribution of genotypes and allele among periodontitis patients and healthy control individuals was not significantly different for the SNP at -607of IL 18 gene.

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